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(21) International Application Number: <b>PCT/US88/02765</b> (22) International Filing Date: <b>10 August 1988 (10.08.88)</b> (31) Priority Application Numbers: <b>084,479 171,693</b> (32) Priority Dates: <b>11 August 1987 (11.08.87) 22 March 1988 (22.03.88)</b> (33) Priority Country: <b>US</b> (71) Applicants: <b>CETUS CORPORATION [US/US]; 1400 Fifty-Third Street, Emeryville, CA 94608 (US). THE STATE OF OREGON acting by and through THE OREGON STATE BOARD OF HIGHER EDUCATION on behalf of THE UNIVERSITY OF OREGON [US/US]; Susan Campbell Hall, University of Oregon Campus, Eugene, OR 97403-1229 (US).</b> (72) Inventors: <b>DRUMMOND, Robert, J. ; 2920 Cindy Court, Richmond, CA 94803 (US). BLOCH, Will ; 421 Liberty Street, El Cerrito, CA 94530 (US). MATTHEWS, Brian, W. ; 3815 Spring Boulevard, Eugene, OR 97405 (US). TOY, Pamela, L. ; 363 Somerset, Oakland, CA 94611 (US). NICHOLSON, Henry, H. ; 1366 Lawrence Street, Eugene, OR 97405 (US).</b>	(74) Agents: <b>MANDEL, Saralynn et al.; Ciotti &amp; Murashige, Irell &amp; Manella, 545 Middlefield Road, Suite 200, Menlo Park, CA 94025 (US).</b> (81) Designated States: <b>AT, AT (European patent), AU, BB, BE (European patent), BG, BJ (OAPI patent), BR, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CM (OAPI patent), DE, DE (European patent), DK, FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, RO, SD, SE, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent).</b>  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(54) Title: <b>PROCARYOTIC XYLOSE ISOMERASE MUTEINS AND METHOD TO INCREASE PROTEIN STABILITY</b>		
(57) Abstract <p>Xylose isomerase (XI) muteins useful in the conversion of glucose to fructose or xylose to xylulose are obtained in usable amounts by protein structural and recombinant DNA methods, including x-ray crystallography, cloning, computer graphic modeling and site-directed mutagenesis and expression of the bacterial DNA sequences encoding native procaryotic xylose isomerase. These native sequences are altered to encode the xylose isomerase muteins having improved catalytic function and/or thermostability, and/or a lowered pH optimum. A method for predicting protein-stabilizing amino acid substitutions is also provided.</p>		

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What is claimed is:

1. A method for increasing the stability of a protein comprising substituting an amino acid at a preselected substitution site in the protein, said substitution site having phi and psi backbone conformational angles in the range of  $\phi = -40^\circ$  to  $-90^\circ$  when  $\psi = 0^\circ$  to  $-60^\circ$ , or in the range of  $\phi = -40^\circ$  to  $-95^\circ$  when  $\psi = 120^\circ$  to  $180^\circ$  and capable of accomodating said amino acid without disruption of the three-dimensional structure of the protein such that introduction of said amino acid decreases the configurational entropy of unfolding of said protein.
2. The method of Claim 1 wherein said preselected substitution site is any amino acid residue except proline and the amino acid introduced at said site is proline, and said method further comprises the step of determining the phi and psi values of the amino acid residue in the amino acid sequence of the protein immediately preceding the side of said proline substitution, such that if the psi value of the preceding amino acid residue is between  $0^\circ$  and  $-90^\circ$  then the substitution site must have phi and psi values in the range of  $\phi = -40^\circ$  to  $-90^\circ$  when  $\psi = 0^\circ$  to  $-60^\circ$ , but if the psi value of the preceding amino acid residue is not between  $0^\circ$  and  $-90^\circ$  the the substitution site may have phi and psi values either in the range of  $\phi = -40^\circ$  to  $-90^\circ$  when  $\psi = 0^\circ$  to  $-60^\circ$ , or in the range of  $\phi = -40^\circ$  to  $-95^\circ$  when  $\psi = 120^\circ$  to  $180^\circ$ .
3. The method of Claim 1 wherein said preselected substitution site is a glycine amino acid residue and the amino acid introduced is any amino acid having a  $\beta$  carbon atom or branched  $\beta$  carbon atom.
4. A method for increasing the stability of a protein comprising substituting a glycine amino acid residue having a

negative phi angle with an alanine to decrease the configurational entropy of unfolding of the protein.

5. A method for selecting substitution sites suitable for introduction of amino acids in a protein such that introduction of said amino acids increases the stability of the protein, comprising the steps of:

a) determining from the crystallographic structure of a protein the backbone conformational angles phi and psi of said protein;

b) screening said phi and psi angles determined in step a) to identify potential substitution sites in said protein having conformational phi and psi angles in the range of  $\phi = -40^\circ$  to  $-90^\circ$  when  $\psi = 0^\circ$  to  $-60^\circ$ , or in the range of  $\phi = -40^\circ$  to  $-95^\circ$  when  $\psi = 120^\circ$  to  $180^\circ$  for introduction of said amino acids; and

c) examining a structural model of the protein to determine from the potential substitution sites identified in step b) substitution sites that will accommodate substitution of an amino acid without disruption of the three-dimensional structure of the protein, whereby substitution of said substitution site results in a decrease in the configurational entropy of unfolding of the protein.

6. The method of Claim 5 wherein the amino acid to be substituted into said substitution site is proline, and the step of screening of step b) comprises the additional substep of determining whether the amino acid residue preceding the potential substitution site identified in step b) has psi angles between  $0^\circ$  and  $-90^\circ$ , and if so then the step c) of examining comprises the substep of determining a substitution site having phi and psi angles in the range  $\phi = -40^\circ$  to  $-90^\circ$  when  $\psi = 0^\circ$  to  $-60^\circ$ .

7. Streptomyces rubiginosus, (S. rubiginosus), xylose isomerase mutein having a change in at least one position in the native amino acid sequence at a position equivalent to a native amino acid residue selected from the group consisting of Lysine<sub>183</sub>, Lysine<sub>289</sub>, Histidine<sub>54</sub>, Histidine<sub>220</sub>, Methionine<sub>223</sub>, Arginine<sub>140</sub>, Tryptophan<sub>16</sub>, Tryptophan<sub>137</sub>, Phenylalanine<sub>94</sub>, Glycine<sub>146</sub>, Glycine<sub>166</sub>, Glycine<sub>197</sub>, Glycine<sub>219</sub>, Glycine<sub>231</sub>, Glycine<sub>248</sub>, Glycine<sub>298</sub>, Glycine<sub>305</sub>, Glycine<sub>369</sub>, Leucine<sub>15</sub>, Alanine<sub>29</sub>, Alanine<sub>33</sub>, Asparagine<sub>107</sub>, Arginine<sub>109</sub>, Glycine<sub>146</sub>, Valine<sub>151</sub>, Glycine<sub>189</sub>, Leucine<sub>192</sub>, Glutamic acid<sub>207</sub>, Arginine<sub>259</sub>, Threonine<sub>342</sub>, Arginine<sub>354</sub>, Glycine<sub>369</sub>, Aspartic acid<sub>28</sub>, Arginine<sub>32</sub>, Serine<sub>64</sub>, Valine<sub>218</sub>, Arginine<sub>292</sub>, Isoleucine<sub>252</sub>, Aspartic acid<sub>9</sub>, Glutamine<sub>21</sub>, Alanine<sub>29</sub>, Arginine<sub>32</sub>, Glutamic acid<sub>38</sub>, Leucine<sub>46</sub>, Aspartic acid<sub>56</sub>, Leucine<sub>58</sub>, Valine<sub>127</sub>, Threonine<sub>133</sub>, Alanine<sub>136</sub>, Arginine<sub>177</sub>, Isoleucine<sub>180</sub>, Leucine<sub>193</sub>, Leucine<sub>211</sub>, Asparagine<sub>227</sub>, Glutamine<sub>234</sub>, Alanine<sub>238</sub>, Leucine<sub>246</sub>, Arginine<sub>284</sub>, Arginine<sub>308</sub>, Leucine<sub>311</sub>, Arginine<sub>316</sub>, Leucine<sub>335</sub>, Valine<sub>362</sub>, Methionine<sub>370</sub>, Leucine<sub>375</sub>, Leucine<sub>383</sub>, Glutamine<sub>21</sub>, Asparagine<sub>92</sub>, Asparagine<sub>107</sub>, Asparagine<sub>185</sub>, Asparagine<sub>227</sub>, Glutamine<sub>234</sub>, Glutamine<sub>256</sub>, Asparagine<sub>309</sub>, Glutamine<sub>377</sub>, Tryptophan<sub>270</sub>, Glycine<sub>146</sub>, Phenylalanine<sub>320</sub>, Histidine<sub>382</sub>, Glutamic acid<sub>337</sub>, Arginine<sub>109</sub>, Glycine<sub>189</sub>, Glutamic acid<sub>144</sub>, Glycine<sub>251</sub>, Glycine<sub>225</sub>, Alanine<sub>366</sub>, Valine<sub>98</sub>, Glutamine<sub>249</sub>, Glycine<sub>219</sub>, Glutamic acid<sub>207</sub>, Aspartic acid<sub>163</sub>, Aspartic acid<sub>57</sub>, Glutamic acid<sub>186</sub>, Glutamic acid<sub>141</sub>, Glutamic acid<sub>221</sub>, Aspartic acid<sub>287</sub>, Arginine<sub>177</sub>; and Aspartic acid<sub>345</sub>.

8. The S. rubiginosus xylose isomerase mutein of Claim 7 wherein the change is in the lysine amino acid residue equivalent to Lys<sub>183</sub> and said change is substitution by an amino acid selected from the group consisting of Arg, Gln, Asn, Asp, Glu, Ser, Thr, His, Tyr, Ala, Val, Leu and Ile; or

the change is in the lysine amino acid residue equivalent to Lysine<sub>289</sub> and said change is substitution by an amino acid selected from the group consisting of Arg, Gln, Asn, Asp, Glu, Ser Thr, His, Tyr, Ala, Val, Leu and Ile; or

the change is in the histidine amino acid residue equivalent to His<sub>54</sub> and said change is substitution by an amino acid selected from the group consisting of Gln, Glu, Asn, Asp, Ser, Thr, Ala, Val, and Tyr; or

the change is in the histidine amino acid residue equivalent to His<sub>220</sub> and said change is substitution by an amino acid selected from the group consisting of Gln, Glu, Asn, Asp, Ser, Thr, Ala, Val, and Tyr; or

the change is in the methionine amino acid residue equivalent to Met<sub>223</sub> and said change is substitution by an amino acid selected from the group consisting of Gly, Ala, Val, Leu, Ile, Phe, Tyr, Gln, and Asn; or

the change is in the arginine amino acid residue equivalent to Arg<sub>140</sub> and said change is substitution by an amino acid selected from the group consisting of Gln, Asn, Glu, Asp, Ile, Leu, Ala, Val, and Tyr; or

the change is in the tryptophan amino acid residue equivalent to Trp<sub>16</sub> and said change is substitution by an amino acid selected from the group consisting of Asn, Gln, Ser, Thr, Gly, Ala, Val, Leu, Ile, Tyr, Phe, and His; or

the change is in the tryptophan amino acid residue equivalent to Trp<sub>137</sub> and said change is substitution by an amino acid selected from the group consisting of Asn, Gln, Ser, Thr, Gly, Ala, Val, Leu, Ile, Tyr, Phe, and His; or

the change is in the phenylalanine amino acid residue equivalent to Phe<sub>94</sub> and said change is substitution by an amino acid selected from the group consisting of Thr, Ser, His, Val, Gly, Ala, Ile, Leu, Asn, and Gln; or

the change is substitution of the glycine amino acid residue equivalent to Gly<sub>x</sub> where x is selected from the group consisting of residues 146, 166, 197, 219, 231, 248, 298, 305 and 369, and said Gly substituted with an amino acid other than glycine; or

the change is substitution by proline in the amino acid residue equivalent to an amino acid residue selected from the group consisting of Leu<sub>15</sub>, Asp<sub>28</sub>, Ala<sub>29</sub>, Arg<sub>32</sub>, Ala<sub>33</sub>, Ser<sub>64</sub>, Asn<sub>107</sub>, Arg<sub>109</sub>, Gly<sub>146</sub>, Val<sub>151</sub>, Gly<sub>189</sub>, Leu<sub>192</sub>, Glu<sub>207</sub>, Val<sub>218</sub>, Ile<sub>252</sub>, Arg<sub>259</sub>, Arg<sub>292</sub>, Thr<sub>342</sub>, Arg<sub>354</sub>, Gly<sub>369</sub>, Arg<sub>177</sub>, and Asp<sub>345</sub>; or

the change is double substitutions of cysteine in the amino acid residues equivalent to pairs of amino acid residues selected from the group consisting of Trp<sub>270</sub> and Gly<sub>146</sub>, Phe<sub>320</sub> and His<sub>382</sub>, Glu<sub>337</sub> and Arg<sub>109</sub>, Gly<sub>189</sub> and Glu<sub>144</sub>, Gly<sub>251</sub> and Gly<sub>225</sub>, Ala<sub>336</sub> and Val<sub>98</sub>, Gln<sub>249</sub> and Gly<sub>219</sub>, and/or Glu<sub>207</sub> and Asp<sub>163</sub>; or

the change is substitution by tyrosine in the amino acid residues equivalent to an amino acid residue selected from the group consisting of Asp<sub>9</sub>, Gln<sub>21</sub>, Ala<sub>29</sub>, Arg<sub>32</sub>, Glu<sub>38</sub>, Leu<sub>46</sub>, Asp<sub>56</sub>, Leu<sub>58</sub>, Val<sub>127</sub>, Thr<sub>133</sub>, Ala<sub>136</sub>, Arg<sub>177</sub>, Ile<sub>180</sub>, Leu<sub>193</sub>, Leu<sub>211</sub>, Asn<sub>227</sub>, Gln<sub>234</sub>, Ala<sub>238</sub>, Leu<sub>246</sub>, Arg<sub>284</sub>, Arg<sub>308</sub>, Leu<sub>311</sub>, Arg<sub>316</sub>, Leu<sub>335</sub>, Val<sub>362</sub>, Met<sub>370</sub>, Leu<sub>375</sub> and Leu<sub>383</sub>; or

the change is substitution by phenylalanine in the amino acid residue equivalent to an amino acid residue selected from the group consisting of Leu<sub>46</sub>, Asp<sub>56</sub>, Leu<sub>58</sub>, Thr<sub>133</sub>, Ala<sub>136</sub>, Ile<sub>180</sub>, Leu<sub>193</sub>, Leu<sub>211</sub>, Asn<sub>227</sub>, Gln<sub>234</sub>, Ala<sub>238</sub>, Leu<sub>246</sub>, Leu<sub>311</sub>, Leu<sub>335</sub>, Val<sub>362</sub>, Met<sub>370</sub>, Leu<sub>375</sub> and Leu<sub>383</sub>; or

the change is substitution by tryptophan in the amino acid residue equivalent to Asn<sub>227</sub>; or

the change is substitution by an amino acid residue selected from the group consisting of Ala, Val, Leu, Ile, Ser, Thr, His, Tyr, Lys, Arg, Met and Pro in the amino acid residue equivalent to an amino acid residue selected from the group consisting of Gln<sub>21</sub>, Asn<sub>92</sub>, Asn<sub>107</sub>, Asn<sub>185</sub>, Asn<sub>227</sub>, Gln<sub>234</sub>, Gln<sub>256</sub>, Asn<sub>309</sub>, and Gln<sub>377</sub>; or

the change is in the aspartic acid amino acid residue equivalent to Asp<sub>57</sub> and said change is substitution by an amino acid selected from the group consisting of Lys, Arg, Gly, Ala, Gln, Asn, Thr and Ser; or

the change is in the glutamic acid amino acid residue equivalent to Glu<sub>186</sub> and said change is substitution by an amino acid selected from the group consisting of Lys, Arg, Gly, Ala, Gln, Asn, Thr and Ser; or

the change is substitution of the aspartic acid amino acid residue equivalent to Asp<sub>57</sub> and said substitution is with an amino acid other than aspartic acid or glutamic acid; or

the change is substitution in the glutamic acid amino acid residue equivalent to Glu<sub>186</sub> and said change is substitution by an amino acid other than aspartic acid or glutamic acid; or

the change is substitution by glutamine in the glutamic acid amino acid residue equivalent to Glu<sub>221</sub>; or

the change is substitution by glutamine in the glutamic acid amino acid residue equivalent to Glu<sub>141</sub>.



9. A nucleic acid encoding the xylose isomerase of Claim 7 or 8 said nucleic acid being substantially free of nucleic acid that does not encode the xylose isomerase of Claim 7 or 8.

10. An expression vector for mutant procaryotic xylose isomerase which comprises the nucleic acid of Claim 9 operably linked to control sequences compatible with a host cell.

11. A method for enhancing the conversion of glucose to fructose and xylose to xylulose which comprises exposing an effective amount of the xylose isomerase mutein of Claim 7 or 8 to glucose and xylose, respectively.

12. The xylose isomerase mutein of Claim 7 wherein the expressed xylose isomerase exhibits a change in one or more of the characteristics of chemical stability,  $k_{catF}$ ,  $k_{catX}$ ,  $K_S$ ,  $K_P$ , temperature stability, specific activity and a lowered pH optimum of the isomerase, as compared to the reference xylose isomerase.

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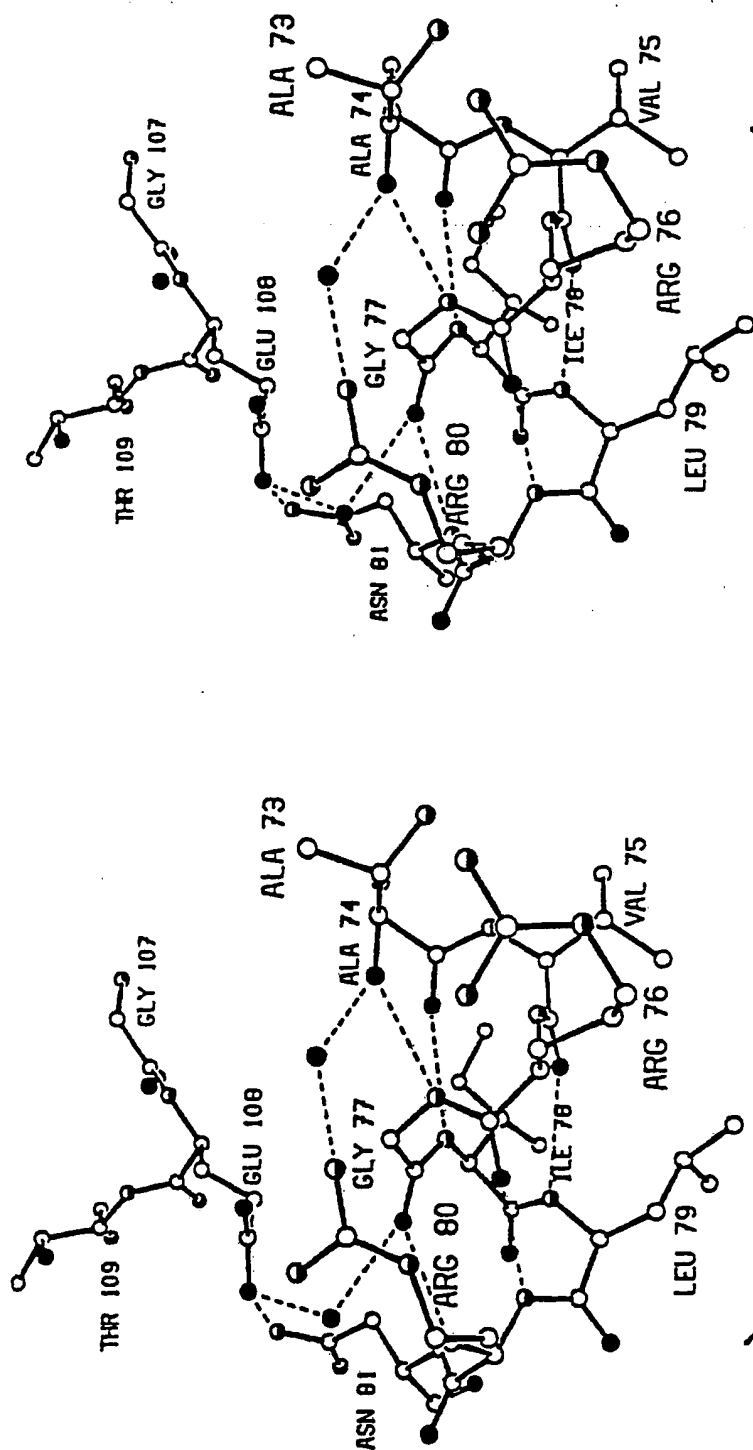


FIG. 1A

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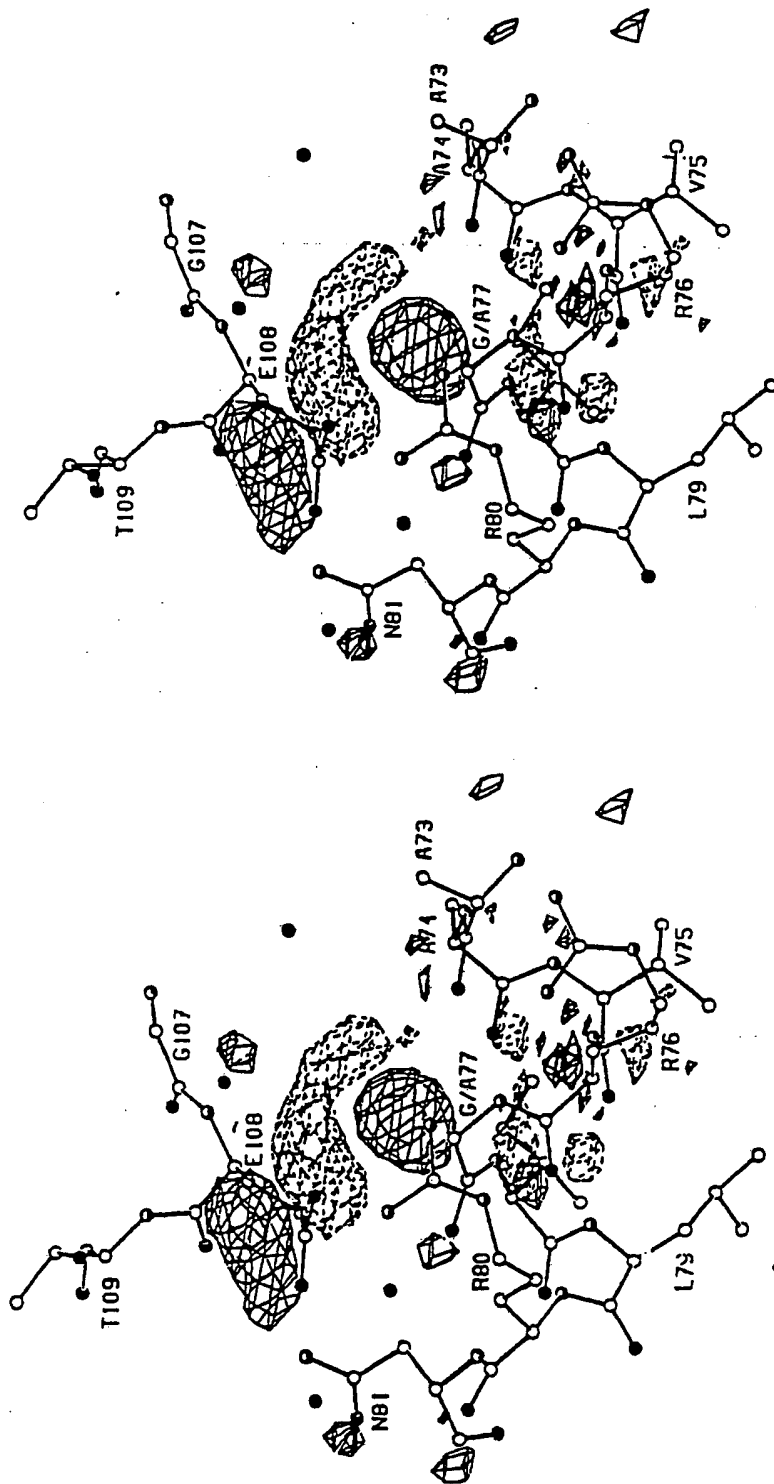


FIG. 1B

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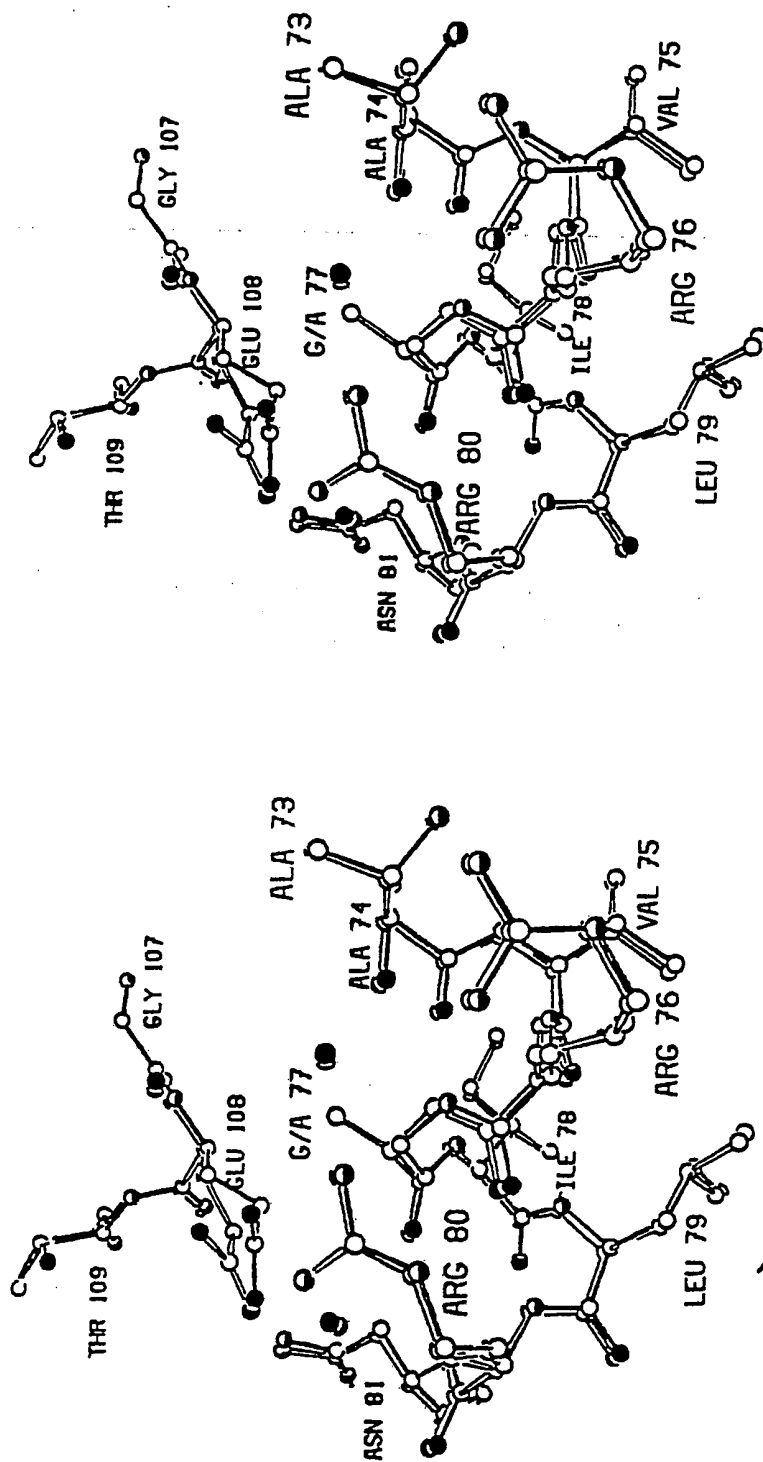
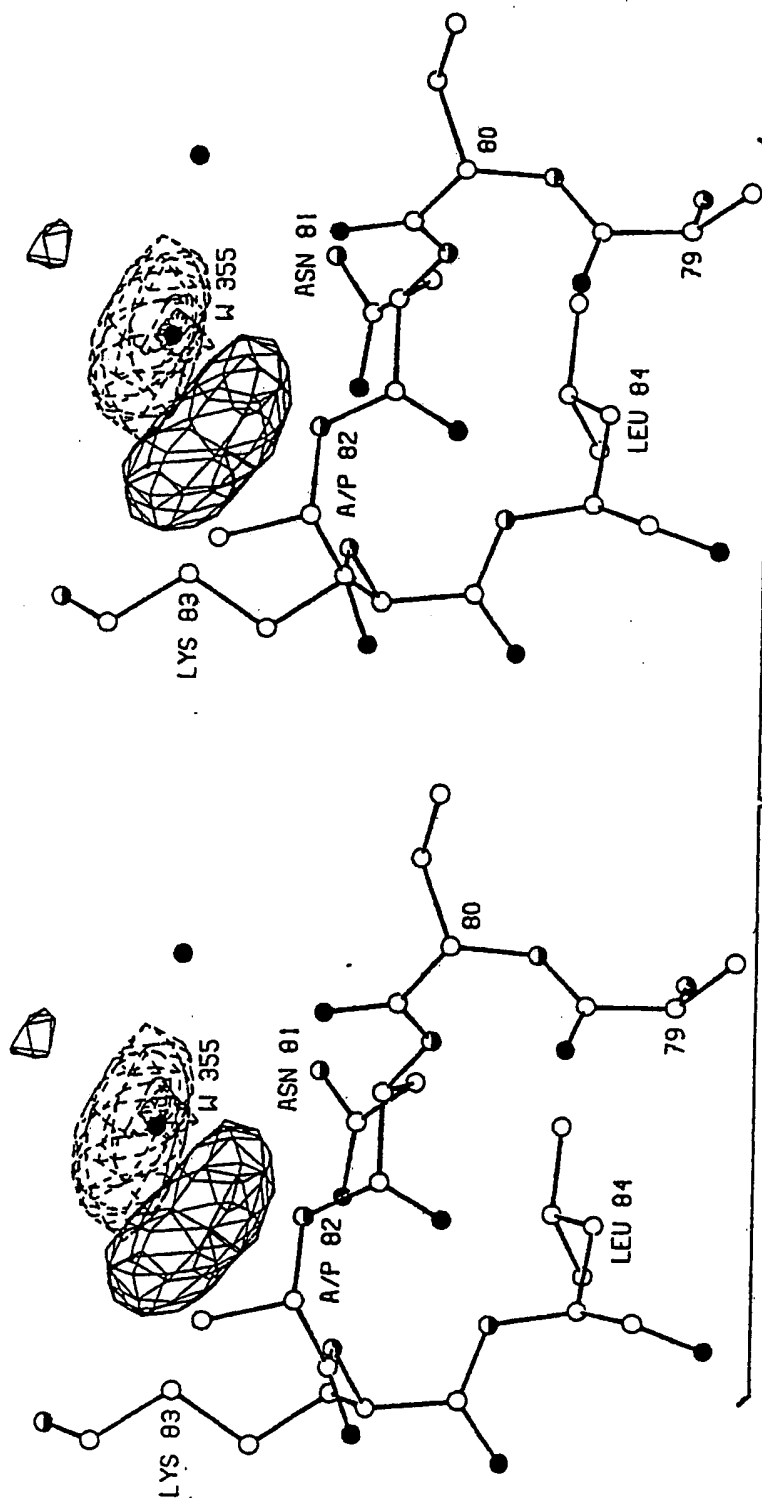


FIG. 1C

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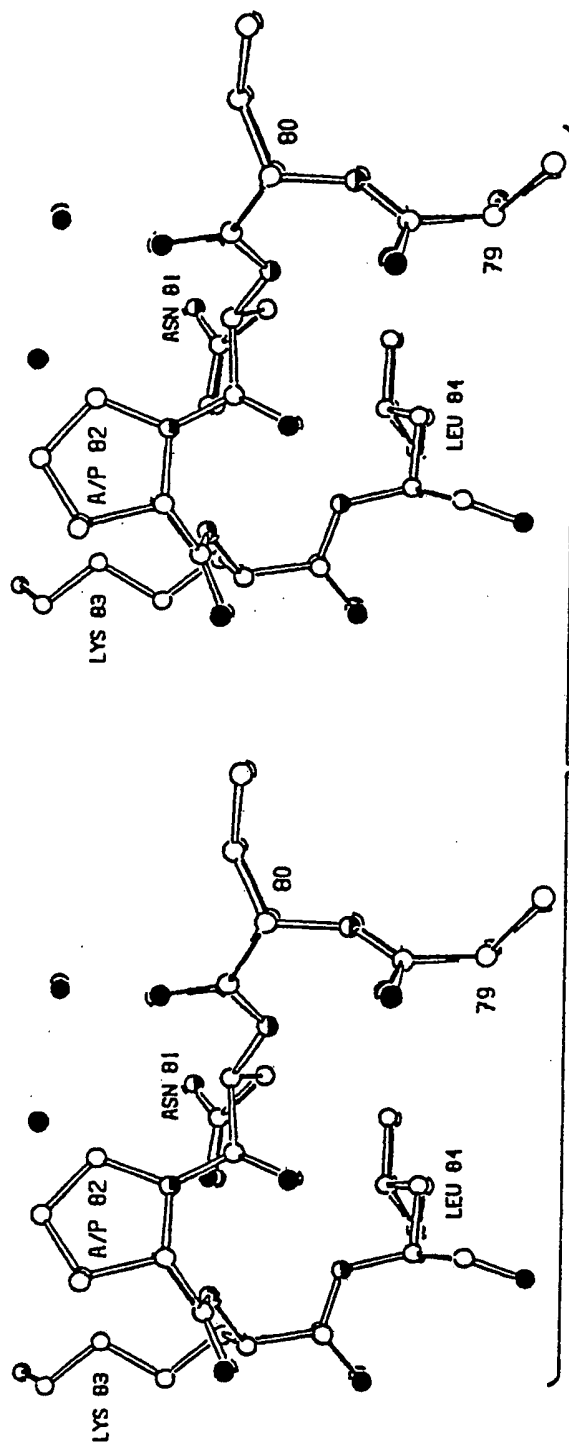


FIG. 2B

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FIG. 3A

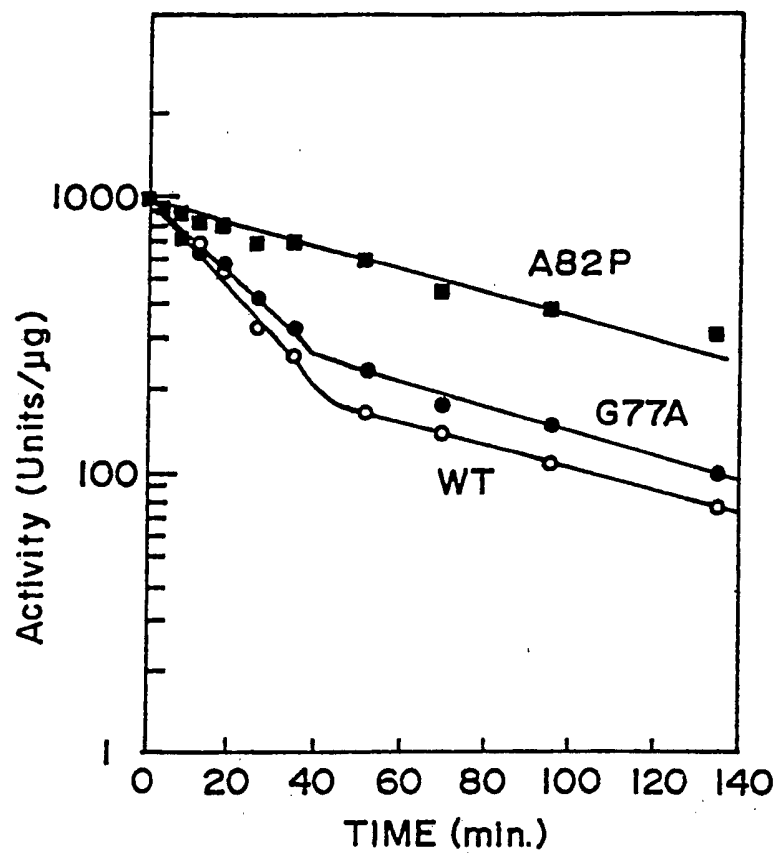
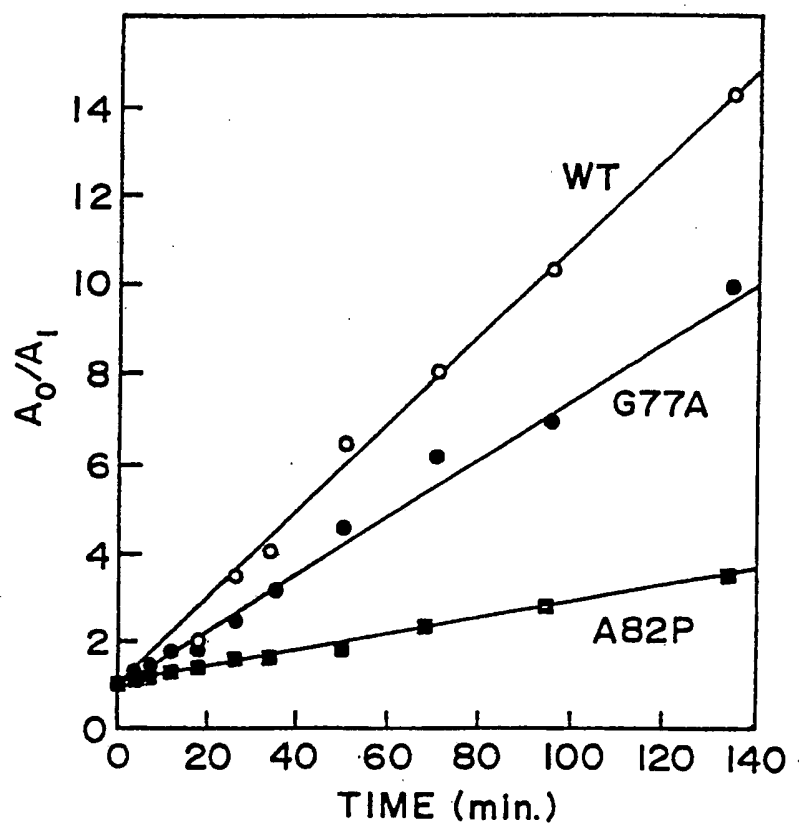


FIG. 3B





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ATGAACTACCAGCCCACCCCCGAGGACAGGTTACCTTCGGACTGTGGACCGTCGGCTGG  
1 METAsnTyrGlnProThrProGluAspArgPheThrPheGlyLeuTrpThrValGlyTrp  
CAGGGACGGGACCCCTTCGGTGACGCCACGCGGCGCGCCCTCGACCCGGTCGAGTCGGTG  
21 GlnGlyArgAspProPheGlyAspAlaThrArgArgAlaLeuAspProValGluSerVal  
CGGCGGCTGGCCGAGCTGGGCGCCACGGCGTCACGTTCCACGACGACGACCTCATCCCC  
41 ArgArgLeuAlaGluLeuGlyAlaHisGlyValThrPheHisAspAspAspLeuIlePro  
TTCGGCTCCAGCGACAGCGAGCGGAGGAGCACGTCAAGCGGTTCCGGCAGGCGCTGGAC  
61 PheGlySerSerAspSerGluArgGluGluHisValLysArgPheArgGlnAlaLeuAsp  
GACACCGGCATGAAGGTGCCGATGGCCACCACCAACCTGTTACCCACCCGGTGTTCAAG  
81 AspThrGlyMETLysValProMETAlaThrThrAsnLeuPheThrHisProValPheLys  
GACGGCGGCTTCACCGCCAACGACCGCGACGTGCGCCGCTACGCCCTGCGCAAGACCATC  
101 AspGlyGlyPheThrAlaAsnAspArgAspValArgArgTyrAlaLeuArgLysThrIle  
CGCAACATCGACCTCGCGGTTCGAGCTCGGCGCCGAGACCTATGTGGCCTGGGGCGGCCGC  
121 ArgAsnIleAspLeuAlaValGluLeuGlyAlaGluThrTyrValAlaTrpGlyGlyArg  
GAGGGTGCCGAGTCGGGTGGCGCCAAGGACGTGCGGGACGCCCTCGACCGCATGAAGGAG  
141 GluGlyAlaGluSerGlyGlyAlaLysAspValArgAspAlaLeuAspArgMETLysGlu  
GCCTTCGACCTGCTCGGCGAGTACGTACCTCCCAGGGCTACGACATCCGCTTCGCCATC  
161 AlaPheAspLeuLeuGlyGluTyrValThrSerGlnGlyTyrAspIleArgPheAlaIle  
GAGCCCAAGCCGAACGAGCCGCGGGCGACATCCTGCTCCCCACCGTCGGCCACGCCCTG  
181 GluProLysProAsnGluProArgGlyAspIleLeuLeuProThrValGlyHisAlaLeu  
GCGTTCATCGAGCGCCTGGAGCGACCGGAGCTGTACGGCGTGAACCCCGAGGTGGCCAC  
201 AlaPheIleGluArgLeuGluArgProGluLeuTyrGlyValAsnProGluValGlyHis  
GAGCAGATGGCCGGGCTGAACTTCCCGCACGGCATCGCGCAGGCGCTGTGGGCGGGCAAG

FIG. 5A

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221 GluGlnMETAlaGlyLeuAsnPheProHisGlyIleAlaGlnAlaLeuTrpAlaGlyLys  
CTGTTCCACATCGACCTCAACGGCCAGAACGGCATCAAGTACGACCAGGACCTCCGCTTC  
241 LeuPheHisIleAspLeuAsnGlyGlnAsnGlyIleLysTyrAspGlnAspLeuArgPhe  
GGCGCGGGCGACCTGCGGGCCGCGTTCTGGCTGGTGGACCTGCTGGAGTCGGCCGGCTAC  
261 GlyAlaGlyAspLeuArgAlaAlaPheTrpLeuValAspLeuLeuGluSerAlaGlyTyr  
AGCGGCCCGCGGCACTTCGACTTCAAGCCGCGCGGACCGAGGACTTCGACGGGGTGTGG  
281 SerGlyProArgHisPheAspPheLysProProArgThrGluAspPheAspGlyValTrp  
GCCTCGGCGGCCGCTGCATGCGCAACTACCTGATCCTCAAGGAGCGTGCGGCGGCCTTC  
301 AlaSerAlaAlaGlyCysMETArgAsnTyrLeuIleLeuLysGluArgAlaAlaAlaPhe  
CGCGCCGACCCCGAGGTGCAGGAGGCGCTGCGCGCGTCCCGTCTGGACGAGCTGGCCCGG  
321 ArgAlaAspProGluValGlnGluAlaLeuArgAlaSerArgLeuAspGluLeuAlaArg  
CCCACGGCGGCCGACGGTCTGCAGGCCCTGCTCGACGACCGGTCCGCCTTCGAGGAGTTC  
341 ProThrAlaAlaAspGlyLeuGlnAlaLeuLeuAspAspArgSerAlaPheGluGluPhe  
GACGTCGACGCGGCGGCGGCCCGTGGGATGGCCTTCGAGCGCCTGGACCAGCTGGCGATG  
361 AspValAspAlaAlaAlaAlaArgGlyMETAlaPheGluArgLeuAspGlnLeuAlaMET  
GACCACCTGCTGGGCGCCCGGGGCTGA  
381 AspHisLeuLeuGlyAlaArgGly...

FIG. 5B

GI  
GI  
GI  
GI

GI  
GI  
GI  
GI  
non

GI  
p GI  
GI  
GI  
mon

GI  
p GI  
GI  
GI  
mon

GI  
GI  
GI  
GI

GI  
GI  
GI  
GI

VGHEQMAAGLNFPPHGGIAQAALWAGKLFHIDDLNGQNGGIGKYYDDQQDDLLRRFGGAGDGLRAAFWLV  
 TGHEQMSNLNFTTQGGIAQAALWAGKLFHIDDLNGQNGGIGKYYDDQQDDLLRRFGGAGDGLRAAFWLV  
 ANHATL A G H T F E H E L R M A R V H G L L G S V D A N Q G H P L L G W D T D E E P T D L Y S T T L A M W  
 ANHATL A G H S F H H E I A T A I A L G L F G S V D A N R G D A Q L G W D T D Q F P N S V E E N A L V M Y

DLLESAGYS--GPRHFDFFKPPRT--EDFDGGVWASAAGCMRNYLILKKERAAAF  
 DLLENGPDDGGPAYDGP R H F D Y K P S R T -- E D F D G G V W E S A K D N I R M Y L L L K K E R A A A F  
 E I L Q N G G L G S -- G G L N F D A K V R R S S F E P D D L V W A H I A G M D A F A R G L K K V A H K --  
 E I L K A G G F T -- G G L N F D A K V R R S S F E P D D L V W A H I A G M D A F A R G L K K I A A R --

RADPEVQE--ALRASRLDELARPTAADGGLQA--LLDD--RRAFEEDFDVDA A A A R G M  
 RADPEVQA--ALAESKVDDELRTPTTLNPGGETYADLLAD--RRAFEEDYDADAVGAKGY  
 -- -- -- -- -- L I E D R V F E D V I Q H R Y R S F T E G I G L E I T E G R A N F H T L E Q Y A L N N K K T I  
 -- -- -- -- -- M I E D G E L D K R I A Q R Y S G W N S E L G Q Q I L K G Q M S L A D L A K Y A Q E H H L S

AFERLDQLAMDHLLGARG--  
 GFVKLNQLAIDHLLGAR--  
 KNE--SGRQERLKPILNQ--  
 PVHQSGRQEQLENLVNHYLFDK

FIG. 6-2

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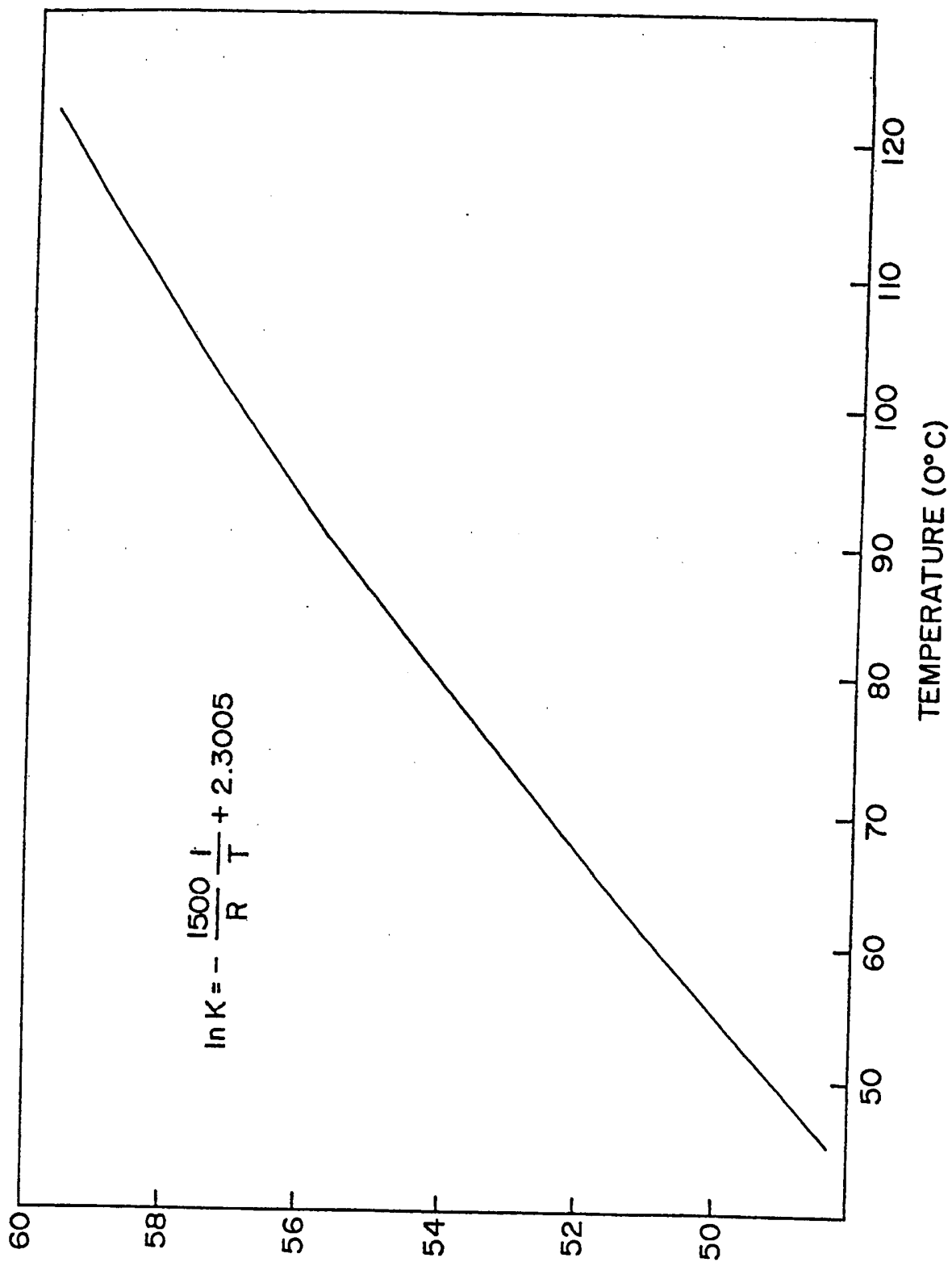
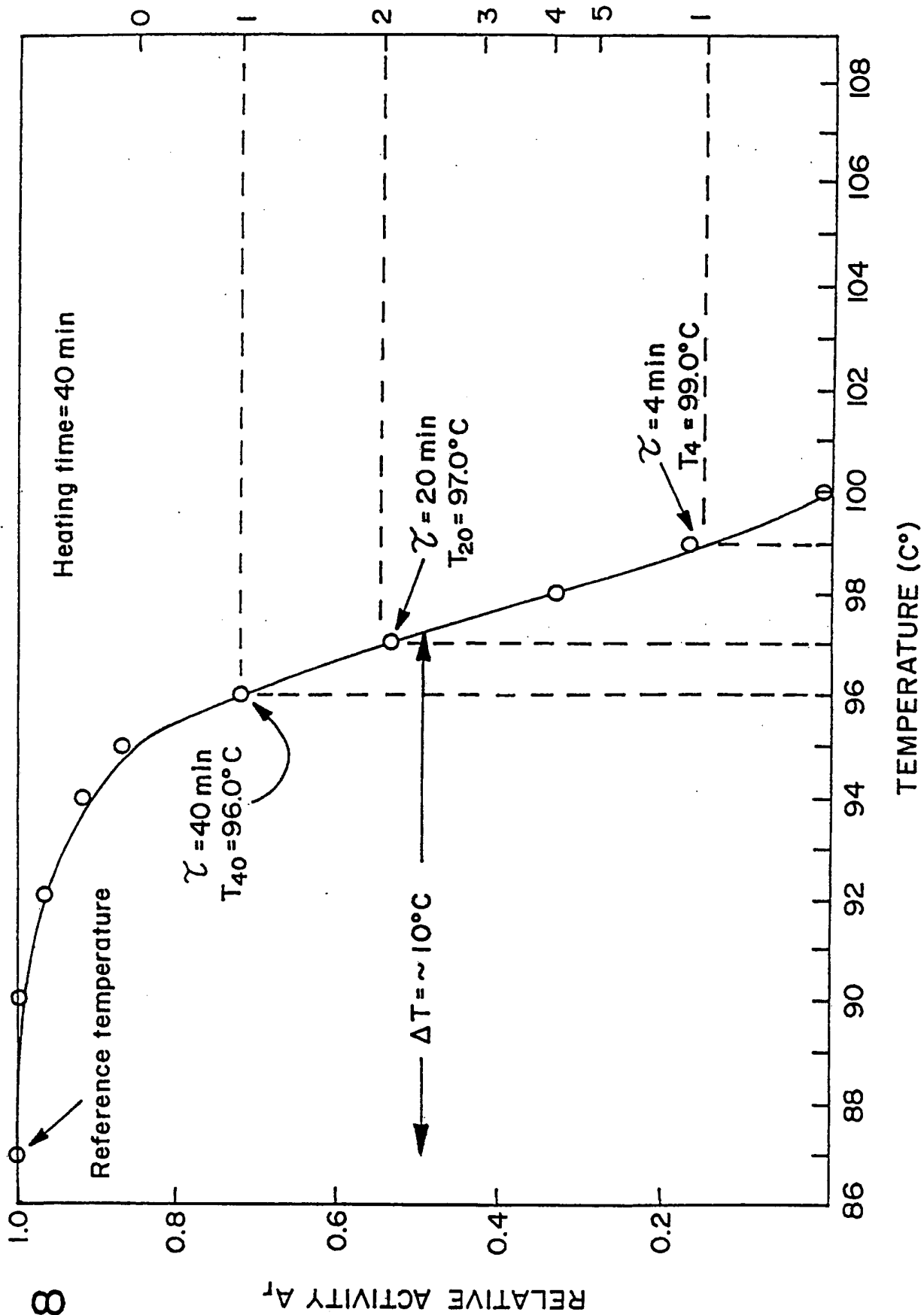


FIG. 7



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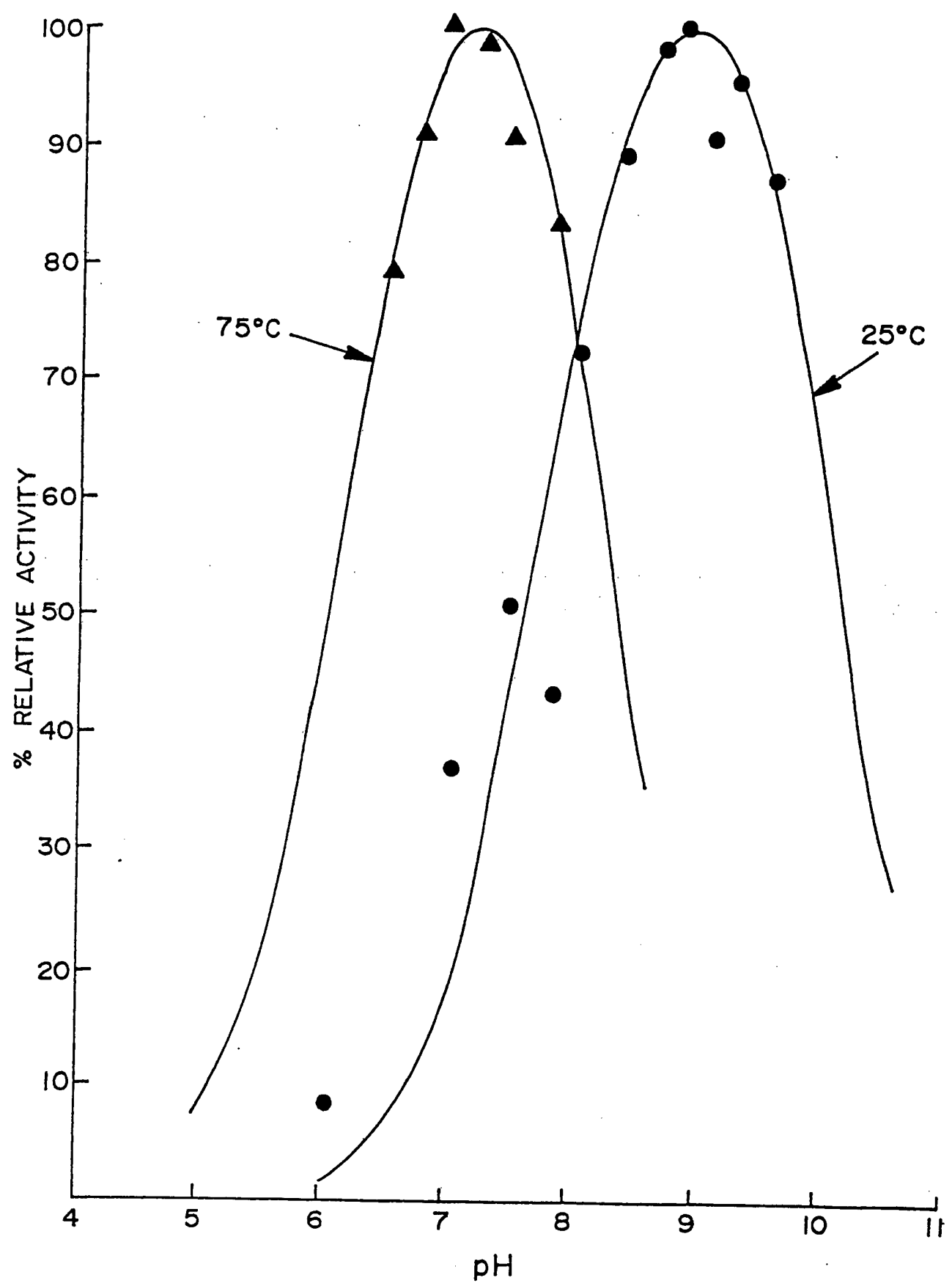


FIG 2

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US88/02765

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC <div style="margin-top: 10px;">           IPC<sup>4</sup>: C 12 N 15/00, C 12 N 9/92, C 12 P 21/02         </div>														
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; margin-top: 10px;">Minimum Documentation Searched <sup>7</sup></div> <table border="1" style="width: 100%; border-collapse: collapse; margin-top: 5px;"> <tr> <td style="width: 30%; padding: 5px;">Classification System</td> <td style="padding: 5px;">Classification Symbols</td> </tr> <tr> <td style="padding: 10px; vertical-align: top;">IPC<sup>4</sup></td> <td style="padding: 10px; vertical-align: top;">C 12 N, C 12 P</td> </tr> </table> <div style="margin-top: 10px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup></div>			Classification System	Classification Symbols	IPC <sup>4</sup>	C 12 N, C 12 P								
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<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>9</sup> <table border="1" style="width: 100%; border-collapse: collapse; margin-top: 5px;"> <tr> <th style="width: 10%; padding: 5px;">Category <sup>10</sup></th> <th style="width: 70%; padding: 5px;">Citation of Document, <sup>11</sup> with Indication, where appropriate, of the relevant passages <sup>12</sup></th> <th style="width: 20%; padding: 5px;">Relevant to Claim No. <sup>13</sup></th> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 10px;">Y</td> <td style="padding: 10px;">           Chemical Abstracts, volume 106, no. 15, 13 April 1987, (Columbus, Ohio, US), Snow, Mark E. et al: "Calculating three-dimensional changes in protein structure due to amino-acid substitutions; the variable region of immunoglobulins", see page 459, abstract 117680v, &amp; Proteins: Struct., Funct., Genet. 1986, 1(3), 267-79 (Eng).  <div style="text-align: center;">--</div> </td> <td style="text-align: center; vertical-align: top; padding: 10px;">1-6</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 10px;">Y</td> <td style="padding: 10px;">           Proc. Natl. Acad. Sci. USA, volume 84, -pp 6663-6667, October 1987, Biochemistry B.W. Matthews et al: "Enhanced protein thermostability from site-directed mutations that decrease the entropy of unfolding", see abstract  <div style="text-align: center;">--</div> </td> <td style="text-align: center; vertical-align: top; padding: 10px;">1-6, 7-8-12</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 10px;">Y</td> <td style="padding: 10px;">           Nature, volume 307, 12 January 1984, pp 187-188 Anthony J. Wilkinson et al: "A large increase in enzyme-substrate affinity  <div style="text-align: center;">-/-</div> </td> <td style="text-align: center; vertical-align: top; padding: 10px;">1-6</td> </tr> </table>			Category <sup>10</sup>	Citation of Document, <sup>11</sup> with Indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	Y	Chemical Abstracts, volume 106, no. 15, 13 April 1987, (Columbus, Ohio, US), Snow, Mark E. et al: "Calculating three-dimensional changes in protein structure due to amino-acid substitutions; the variable region of immunoglobulins", see page 459, abstract 117680v, & Proteins: Struct., Funct., Genet. 1986, 1(3), 267-79 (Eng). <div style="text-align: center;">--</div>	1-6	Y	Proc. Natl. Acad. Sci. USA, volume 84, -pp 6663-6667, October 1987, Biochemistry B.W. Matthews et al: "Enhanced protein thermostability from site-directed mutations that decrease the entropy of unfolding", see abstract <div style="text-align: center;">--</div>	1-6, 7-8-12	Y	Nature, volume 307, 12 January 1984, pp 187-188 Anthony J. Wilkinson et al: "A large increase in enzyme-substrate affinity <div style="text-align: center;">-/-</div>	1-6
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Δ" document member of the same patent family</p> </div> </div>														
<b>IV. CERTIFICATION</b> <table border="1" style="width: 100%; border-collapse: collapse; margin-top: 5px;"> <tr> <td style="width: 50%; padding: 5px;">           Date of the Actual Completion of the International Search  <div style="margin-top: 10px; text-align: center;">1st December 1988</div> </td> <td style="width: 50%; padding: 5px;">           Date of Mailing of this International Search Report  <div style="margin-top: 10px; text-align: center;">28 DEC 1988</div> </td> </tr> <tr> <td style="width: 50%; padding: 5px;">           International Searching Authority  <div style="margin-top: 10px; text-align: center;">EUROPEAN PATENT OFFICE</div> </td> <td style="width: 50%; padding: 5px;">           Signature of Authorized Officer  <div style="margin-top: 10px; text-align: center;"> <b>P.C.G. VAN DER PUTTEN</b> </div> </td> </tr> </table>			Date of the Actual Completion of the International Search <div style="margin-top: 10px; text-align: center;">1st December 1988</div>	Date of Mailing of this International Search Report <div style="margin-top: 10px; text-align: center;">28 DEC 1988</div>	International Searching Authority <div style="margin-top: 10px; text-align: center;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="margin-top: 10px; text-align: center;"> <b>P.C.G. VAN DER PUTTEN</b> </div>								
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## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
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Y	Chemical Abstracts, volume 90, no. 9, 26 February 1979, (Columbus, Ohio, US), Creighton, Thomas E.: "Possible im- plications of many proline residues for the kinetics of protein unfolding and refolding", see page 160, abstract 68016y, & J.Mol.Biol. 1978, 125(3), 401-6 (Eng)	1-6
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Y	Research Article, 19 April 1985, p 291 Charles S. Craik et al: "Redesigning Trypsin: Alteration of Substrate Specificity", see whole document,	1-6 7-8,12
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Y	US, A, 4 410 627 (NORMAN E. LLOYD et al) 18 October 1983 see column 7, lines 51-52	7-12
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Y	Chemical Abstracts, volume 100, no. 17, 23 April 1984, (Columbus, Ohio, US), Carrell, H.L. et al: "X-ray crystal structure of D-xylose isomerase at 4-A resolution", abstract 135020k, & J. Biol. Chem. 1984, 259(5), 3230-6 (Eng)	7-12
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Y	Chemical Abstracts, volume 108, no. 15, 11 April 1988, (Columbus, Ohio, US), Farber, Gregory K et al: "The 3.0 A crystal structure of xylose isome- rase from Streptomyces olivochromoge- nes", abstract 127608h, & Protein Eng. 1987, 1(6), 459-66 (Eng)	7-12
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Y	Chemical Abstract, volume 108, no. 17, 25 April 1988, (Columbus, Ohio, US), Henrick, K. et al: "Comparison of backbone structures of glucose isome- rase from Streptomyces and Arthrobac- ter", abstract 146202n, & Protein Eng. 1987, 1(6), 467-9 (Eng)	7-12
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ANNEX TO THE INTERNATIONAL SEARCH REPORT PCT/US88/02765  
ON INTERNATIONAL PATENT APPLICATION NO. SA 24149

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office (EPO) file on 02/11/88. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 4410627	18-10-83	BE-A- 897165	29-12-83
		GB-A- 2123001	25-01-84
		FR-A- 2529572	06-01-84
		SE-A- 8303725	31-12-83
		DE-A- 3323617	19-01-84
		NL-A- 8302334	16-01-84
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		CA-A- 1200521	11-02-86
		AU-A- 558696	05-02-87
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EP-A- 0068647	05-01-83	JP-A- 58031988	24-02-83
		US-A- 4393137	12-07-83
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## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	EP, A2, 0 068 647 (THE UPJOHN COMPANY) 5 January 1983 whole document  -----	7-12